

Competitive titration in living sea urchin embryos of regulatory factors required for expression of the *CyIIIa* actin gene

ROBERTA R. FRANKS, ROGER ANDERSON, JAMES G. MOORE, BARBARA R. HOUGH-EVANS, ROY J. BRITTEN and ERIC H. DAVIDSON

Division of Biology, California Institute of Technology, Pasadena, CA 91125, USA

Summary

Previous studies have located some twenty distinct sites within the 2.3 kb 5' regulatory domain of the sea urchin *CyIIIa* cytoskeletal actin gene, where there occur *in vitro* high-specificity interactions with nuclear DNA-binding proteins of the embryo. This gene is activated in late cleavage, exclusively in cells of the aboral ectoderm cell lineages. In this study, we investigate the functional importance *in vivo* of these sites of DNA–protein interaction. Sea urchin eggs were coinjected with a fusion gene construct in which the bacterial chloramphenicol acetyltransferase (CAT) reporter gene is under the control of the entire *CyIIIa* regulatory domain, together with molar excesses of one of ten nonoverlapping competitor *subfragments* of this domain, each of which contains one or a few specific site(s) of interaction. The exogenous excess binding sites competitively titrate the available regulatory factors away from the respective sites associated with the *CyIIIa* · CAT reporter gene. This provides a method for detecting *in vivo* sites within the regulatory

domain that are required for normal levels of expression, without disturbing the structure of the regulatory domain. We thus identify five nonoverlapping regions of the regulatory DNA that apparently function as binding sites for positively acting transcriptional regulatory factors. Competition with a subfragment bearing an octamer site results in embryonic lethality. We find that three other sites display no quantitative competitive interference with *CyIIIa* · CAT expression, though as shown in the accompanying paper, two of these sites are required for control of spatial expression. We conclude that the complex *CyIIIa* regulatory domain must assess the state of many distinct and individually necessary interactions in order to properly regulate *CyIIIa* transcriptional activity in development.

Key words: sea urchin embryo, regulatory factors, *in vivo*, *CyIIIa* actin gene.

Introduction

The *CyIIIa* cytoskeletal actin gene of the sea urchin *Strongylocentrotus purpuratus* is transcriptionally activated late in cleavage in the presumptive aboral ectoderm territory of the embryo and, throughout embryonic development, the gene continues to be transcribed exclusively in the aboral ectoderm (Shott *et al.* 1984; Cox *et al.* 1986; Lee, 1986; Hickey *et al.* 1987). A necessary and sufficient *cis*-regulatory domain extending approximately 2.3 kb upstream of the *CyIIIa* transcription start site, which promotes correct temporal and spatial activation of a covalently associated CAT reporter gene, has been identified by gene transfer experiments (Flytzanis *et al.* 1987; Hough-Evans *et al.* 1987, 1988; reviewed by Davidson, 1989). The *CyIIIa* *cis*-regulatory domain includes about 20 sites where there occur *in vitro* high-specificity DNA–protein interactions. These have been located in gel shift mapping experiments with specific oligonucleotide competitors, using crude embryonic nuclear extracts (Calzone *et al.*

1988; Thézé *et al.* 1990). This set of sites includes 13 wholly non-homologous, distinct recognition sequences, some of which occur several times in the *CyIIIa* regulatory domain. There are unlikely to be additional binding sites in any of the fragments used in the competition experiments beyond those already characterized, that is, for factors that display a meaningful level of specific binding *in vitro*. Our objective in the present work was to obtain evidence *in vivo* of the functional significance of individual *CyIIIa* protein-binding sites, or of small subsets of locally contiguous sites. The approach chosen was based on the prior *in vivo* competition experiments of Livant *et al.* (1988). There it was shown that coinjection with the *CyIIIa* · CAT fusion of low (2–20×) molar excesses of the whole regulatory domain *per se* (i.e. lacking the CAT reporter gene) resulted in a near stoichiometric, quantitative decrease in reporter gene activity as a function of regulatory sequence:CAT reporter fusion gene ratio (molar excess, as used here, refers to the number of copies of competitor sites compared to

CyIIIa·CAT genes.) In the studies described in this and the accompanying paper, we coinjected restriction fragments containing *subregions* of the regulatory domain, together with a near saturating number of complete CyIIIa·CAT genes. The significance of individual target sites (or small sets of target sites) on these subfragments was determined by assessing the activity of the CAT reporter gene later in development. From this approach two forms of information can be derived. When the coinjected restriction fragments contain sites of positive DNA–factor interaction that *in vivo* are required for normal gene function, competition for these specific factors depresses the level of CAT activity measured in the transgenic embryos. In the accompanying paper, we show that ectopic spatial expression results from competition with other specific regulatory target sequences, implying that the interactions occurring in these sequences function *in vivo* to control spatial expression.

Materials and methods

Preparation and injection of *S. purpuratus* eggs

Unfertilized eggs were microinjected as previously described (McMahon *et al.* 1985). Briefly, the eggs were dejellied by exposure to pH 5.0 filtered sea water (FSW) and fixed electrostatically to protamine sulfate-coated dishes. Approximately 2 µl of a DNA solution was injected into the cytoplasm of each egg. After injection the eggs were fertilized and cultured at 15°C, in the same dishes, in FSW containing penicillin (20 units ml⁻¹) and streptomycin (50 µg ml⁻¹).

CAT fusion genes and whole regulatory domain competitors

Two CyIIIa·CAT fusion genes were used in these experiments. The 14 kb CyIIIa·CAT construct (Flytzanis *et al.* 1987) contains about 7 kb of genomic DNA extending 5' from the transcription initiation site of the *Strongylocentrotus purpuratus* CyIIIa cytoskeletal actin gene, plus the 5' leader sequence of the CyIIIa primary transcript that is interrupted by a 2.2 kb intron (Akhurst *et al.* 1987). The fusion point is a *SalI* site located 11 codons following the start codon of the CyIIIa·CAT message. An SV40 poly(A) addition site and plasmid sequences are also included. The CyIIIa·CAT construct was linearized for microinjection at a unique *SphI* site located about 2.5 kb upstream of the transcription start site (see Fig. 1). A second fusion gene was created by in-frame subcloning the 9.5 kb *SalI* fragment from CyIIIa·CAT (Fig. 1) into the CAT vector pUC.PL·CAT gene (Bond-Matthews and Davidson, 1988). The vector was first modified to create a *SalI* site (R. Franks *et al.* unpublished; Sucov *et al.* 1988). This fusion is called pCyIIIa·CAT·1. The 6.3 kb *SphI*–*KpnI* fragment from CyIIIa·CAT·1 was gel purified and used for microinjection (Fig. 1). This fragment includes all necessary CyIIIa regulatory sequences.

Subfragments of the CyIIIa regulatory domain used as competitors

Subfragments that were used for the *in vivo* competition experiments were derived either directly from the CyIIIa·CAT construct, or from pUC 18/19 subclones of the CyIIIa regulatory region (Calzone *et al.* 1988; F. Calzone, R. Hill, E.

Davidson, unpublished), or equivalent Bluescript subclones (A. Cutting and E. Davidson, unpublished) by digestion with the appropriate restriction enzymes (see legend to Fig. 3). The fragments were gel purified. Subfragments less than approximately 1.0 kb in length were ligated *in vitro* to sperm DNA (prepared as previously described; Lee *et al.* 1984). The sperm DNA was digested with an appropriate restriction enzyme to produce molecules of an average length of about 5.0 kb, the termini of which would be homologous with the competitor DNA termini, or blunt-ended if the competitor DNA molecule bore a blunt end. Ligation reactions were performed at 15°C overnight in 20–50 µl that contained up to 10 µg of total DNA with an average molar ratio of competitor DNA fragments/carrier DNA fragments of about 5.0. The ligated DNA was digested with *BglII*, an enzyme for which none of the competitor DNA subfragments included a site, and molecules ranging in size from approximately 1 kb to 15 kb were obtained for microinjection by fractionation in a 1% low-melt agarose gel, followed by purification by the GeneClean (Bio 101 Inc.) procedure. Alternatively, the *BglII*-digested DNA was size-fractionated by gel filtration through Bio-Gel A-5m (BioRad). The actual concentration of the specific competitor DNA sequence in the final sample used for injection was measured by slot-blot hybridization, using a single-stranded RNA probe. Known numbers of linearized CyIIIa·CAT DNA molecules were included on each filter as standards. Typically, the competitor DNA sequence was present at an average density of about 3.4 copies per 5 kb of carrier DNA in the sample (all samples used fell within the range 2–6 copies per 5 kb carrier DNA except the P71 ligation, which was injected at 1 copy per 5 kb). A few deviations are noted in the legend to Fig. 3.

Microinjection solutions

Each DNA solution was made up in 40% glycerol and contained 550–2000 molecules of a CAT fusion gene per µl, together with competitor DNA and/or carrier DNA (see text). The DNA mass in each injection solution was adjusted by the addition of carrier to yield at least a 5-fold mass excess of carrier DNA plus competitor DNA, over CAT DNA, and to ensure that each injection sample in a given experiment contained the same total DNA concentration.

Measurement of CAT enzyme content per embryo

Embryos were analyzed for CAT enzyme content at 20–24 h postfertilization (hatched blastula) as previously described (McMahon *et al.* 1984), using lysates derived from 40–60 injected embryos and a series of bacterial CAT enzyme standards. Under our conditions, one unit of CAT enzyme activity equals $\sim 2.6 \times 10^{11}$ molecules of enzyme, as established earlier by reference to a purified recombinant CAT enzyme preparation (McMahon *et al.* 1984).

Measurement of CAT DNA and competitor DNA content per embryo

DNA measurements were made at 24 h postfertilization by a modification of the slot-blot procedure described earlier (Flytzanis *et al.* 1987; Franks *et al.* 1988). Centrifugal pellets containing 60–130 injected embryos or uninjected control embryos were mixed in 50 µl of 250 mM Tris–HCl, pH 7.8 with ~ 100 gastrula stage *Lytechinus variegatus* embryos used as carrier. An equal volume of 0.1 M EDTA, pH 8.0, 0.2 M NaCl, 1% SDS was added, and the samples were incubated with 20 µg of proteinase K for 3 h at 55°C. The nucleic acids were extracted and treated with 0.4 M NaOH at 65°C to denature the DNA and hydrolyze the RNA, and were filtered onto

nitrocellulose using a slot-blot apparatus as previously described. For hybridization standards known numbers of CyIIIa·CAT DNA molecules, linearized with *Sph*I, were mixed with *L. variegatus* carrier nucleic acids, treated with 0.4 M NaOH as described above, and applied to each filter. These standards of course include all of the different competitive sequences, since the whole regulatory domain is present in CyIIIa·CAT (see Fig. 1) as well as the CAT gene sequence. The slots were cut exactly in half, and the halves were separately hybridized with single-stranded RNA probes containing sequences representing either the CAT gene (Flytzanis *et al.* 1987) or the CyIIIa 5'-sequence used for *in vivo* competition. Probe specific activities were about 1.3×10^9 cts min⁻¹ µg⁻¹. Hybridizations with the CAT probe were carried out at 40°C in 50% formamide as described (Franks *et al.* 1988). Hybridizations with the CyIIIa 5'-competitor sequence probes were performed at temperatures calculated to maximize hybridization efficiency based on G-C content (Casey and Davidson, 1977). The filters were washed twice with 2×SSC (1×SSC is 0.15 M NaCl, 0.015 M sodium citrate), 0.2% SDS at room temperature, twice with 2×SSC, 0.2% SDS at 68°C, and twice with 0.75×SSC, 0.2% SDS at 68°C. Following autoradiography, each half-slot was cut out and counted; quantitation of CAT DNA sequences or CyIIIa 5'-competitor DNA sequences in each half-slot was made by comparison to the signal obtained for hybridization between each probe and the CyIIIa·CAT DNA standards on each filter. The competitor DNA sequence probes also hybridized with the incorporated CyIIIa·CAT sequences present in each half-slot of DNA extracted from the injected embryos. Therefore, the number of competitor DNA molecules in excess of CyIIIa·CAT genes in a given half-slot could be determined by subtracting the number of CyIIIa·CAT DNA molecules that was calculated to be present (using the corresponding half-slot hybridization signal obtained with the CAT probe) from the total number of sequences that hybridized with the competitor DNA probe. Thus the molar ratio of incorporated competitor DNA: CyIIIa·CAT DNA in the transgenic embryos was obtained. The number of CAT DNA molecules and competitor DNA molecules present per half-slot was also computed in terms of average numbers of molecules per embryo.

Competitive efficiency coefficient α

The competitive *in vivo* titrations carried out with the whole regulatory domain by Livant *et al.* (1988) indicated that the efficiency of competition *in vivo* was about 44% of ideal expectation. This was established by deriving a competition efficiency coefficient, α , given the form $y = (1 + \alpha x)^{-1}$, where y is the CAT activity relative to the control, in which CyIIIa·CAT but no competitor DNA is introduced into the embryos; and x is the molar ratio of competitor to CyIIIa·CAT DNA actually incorporated in the experimental embryos. Were $\alpha = 1$, the factor binding sites included in the incorporated CyIIIa·CAT genes, and those in the incorporated competitor regulatory domains, would have competed with equal efficiency for the limiting DNA binding factors. The microinjection gene transfer system utilized in both the experiments of Livant *et al.* (1988) and those described in this paper, produces a mosaic, though random, pattern of exogenous DNA incorporation (McMahon *et al.* 1985; Flytzanis *et al.* 1985; Hough-Evans *et al.* 1987; Franks *et al.* 1988; see Livant *et al.* 1988). Thus, a probable explanation for the ~2-fold discrepancy from ideal behavior in the experiments of Livant *et al.* (1988) is that the cells incorporating and expressing CyIIIa·CAT in their genomes are not invariably the same cells that incorporate all the competitor sequences.

Results

Competitive titration using the entire CyIIIa regulatory domain

Previously published gene transfer studies demonstrating that correct spatial and temporal expression is mediated by upstream CyIIIa sequence were carried out with the linearized CyIIIa·CAT fusion (Flytzanis *et al.* 1987; Hough-Evans *et al.* 1987, 1988), and this construct was also used in some of the experiments we report here. A second construct, pCyIIIa·CAT·1, utilized in other experiments, is shown diagrammatically in Fig. 1A. Expression of the 6.3 kb *Sph*I–*Kpn*I fragment of pCyIIIa·CAT·1 in transgenic embryos is indistinguishable from that of CyIIIa·CAT in quantitative, temporal, and spatial respects. On microinjection into sea urchin egg cytoplasm, linear DNA molecules of these lengths are rapidly ligated, irrespective of the DNA termini, into long end-to-end concatenates that are stably incorporated into one or more blastomere nuclei during cleavage, and are replicated together with host cell DNA throughout embryogenesis (McMahon *et al.* 1985; Flytzanis *et al.* 1985). No differences were detected in the degree of amplification of any of the fusion genes or competition fragments within experiments.

In an initial series of experiments, fragments containing the complete set of detectable protein-binding sequence elements were used as *in vivo* competitors in order to provide base line stoichiometry for comparison with the competitive effects of individual subfragments. In Fig. 2 we present new data that substantiate the observation (Livant *et al.* 1988) that quantitative competitive titrations can be obtained *in vivo* in sea urchin embryos. This Fig. shows the pooled 'whole regulatory domain' controls for the subfragment competition experiments presented in the following. For each point in Fig. 2, at least 1200 molecules of the CyIIIa·CAT reporter, plus the indicated numbers of competitive DNA molecules (9.5 kb *Sal*I or 4.3 kb *Nco*I–*Eco*RI fragments, see Fig. 1) were injected. At least a 5-fold mass excess of carrier and/or competitor DNA was coinjected with the CyIIIa·CAT genes. The carrier used was sea urchin sperm DNA, cut to a mean length of 5 kb. Thus the individual CyIIIa·CAT genes in the incorporated concatenates were distributed around a mean distance apart of 30–70 kb (depending on whether the 14 kb CyIIIa·CAT or the 6.3 kb *Sph*I–*Kpn*I fragment was used), interspersed among randomly selected sequences; or, in the competition samples, among various ratios of CyIIIa regulatory domain and 5 kb randomly selected sequence. Preliminary experiments had shown that the CyIIIa·CAT gene functions 2–3× more efficiently when separated by ≥3.5-fold excess of carrier (possibly due to transcriptional interference in the absence of carrier). Within each experimental series of Fig. 2, the various competition samples and the reference sample, in which CyIIIa·CAT and carrier but no competitor had been introduced, all received exactly the same total mass of DNA per egg; i.e. the carrier DNA content was adjusted to take into account the

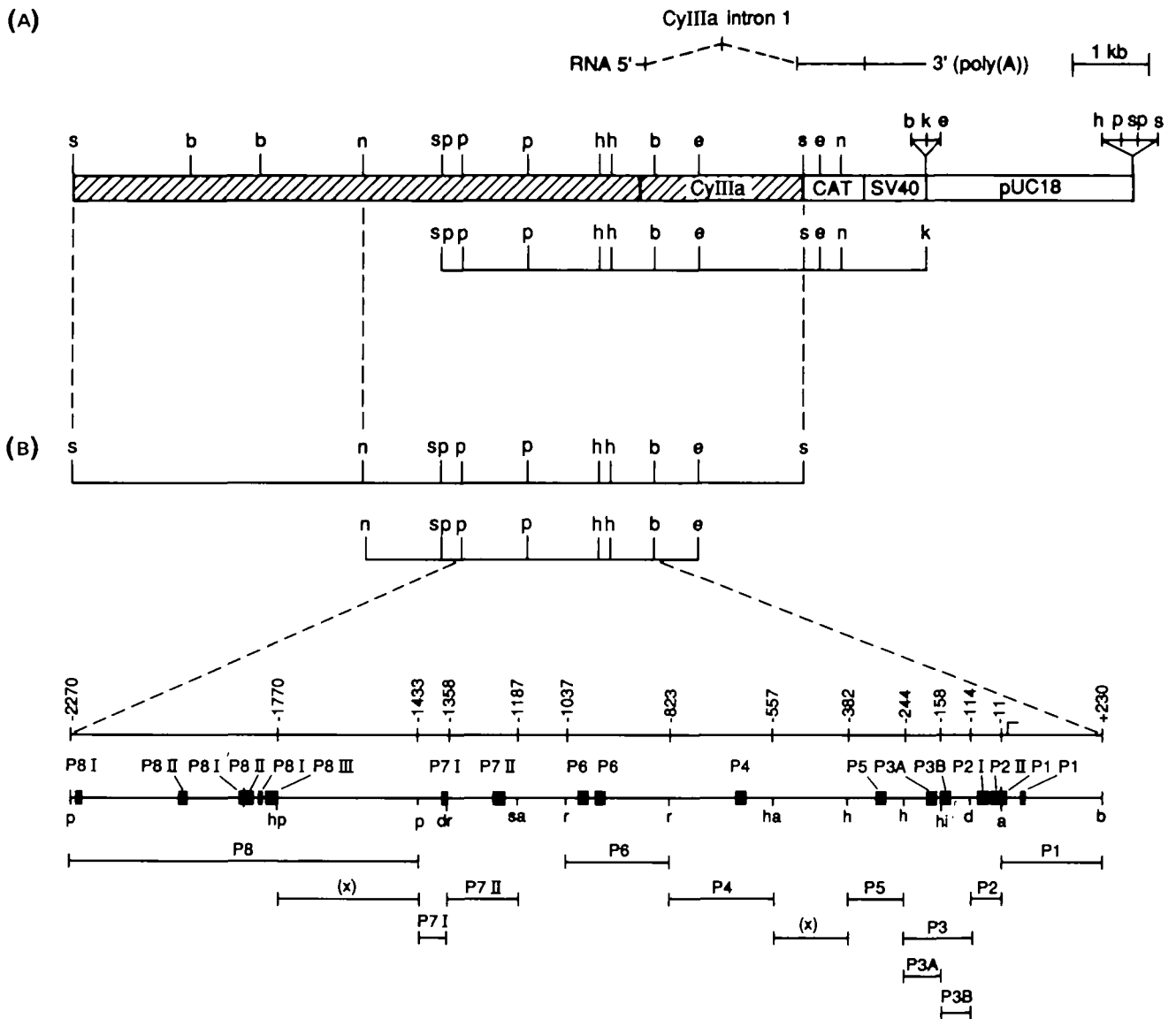


Fig. 1. Structure of the pCyIIIa · CAT · 1 gene fusion, locations of sites of *in vitro* interaction between nuclear factors and CyIIIa 5'-flanking sequence, and competitor DNA fragments. (A) Map of pCyIIIa · CAT · 1. Hatched areas represent sea urchin sequences, including the 2.2 kb first intron contained within the CyIIIa primary transcript (Akhurst *et al.* 1987). The fusion point is a *SalI* site located 11 codons following the start codon of pCyIIIa · CAT · 1. An SV40 poly(A) addition site is included, together with plasmid sequences. The 6.3 kb *SphI*–*KpnI* fragment derived from the pCyIIIa · CAT · 1 fusion gene was used for microinjection. Alternatively, the CyIIIa · CAT fusion gene (not shown) described by Flytzanis *et al.* (1987) was linearized at the *SphI* site located 2.5 kb upstream of the CyIIIa transcription start site, and used for microinjection. (B) Map of the CyIIIa cis-regulatory domain, and competitor DNA fragments. The locations of all known sites of *in vitro* interaction between sequence elements in the 5'-flanking regulatory region of the CyIIIa gene and nuclear factors are depicted by black boxes within the 2.5 kb *PstI*–*BamHI* fragment. The protein–DNA interactions described by Thézé *et al.* (1990) for each binding site are indicated (P1–P8). The P1 binding region actually extends into the 5' end of the first exon of the CyIIIa gene. The 9.5 kb CyIIIa *SalI* fragment and the 4.3 kb *NcoI*–*EcoRI* fragment (indicated above the map), each of which contains the entire regulatory domain, were used as competitor in the *in vivo* competition experiments shown in Fig. 2. DNA subfragments of the regulatory region (shown below the map) that contain the indicated factor-binding site(s) (or no binding site, x) were released from pUC subclones (Calzone *et al.* 1988) by digestion from the polylinker with *EcoRI* and *HindIII* (P1–P3, P4–P6, x) or *HindIII* (P5), or from pBluescript subclones (A. Cutting, E. Davidson, unpublished data) by digestion with *BamHI* and *HindIII* (P3A, P7I). Fragment P3B was derived from fragment P3 by digestion with *HinfI*. These fragments were ligated to sea urchin carrier DNA molecules bearing termini homologous with the specific fragment and prepared for microinjection as described in Materials and methods, and were subsequently used for the *in vivo* competition experiments shown in Fig. 3 and Table 1. Restriction sites shown are: a, *AvaII*; b, *BamHI*; d, *DdeI*; dr, *DraI*; h, *HindIII*; ha, *HaeIII*; hi, *HinFI*; hp, *HpaII*; k, *KpnI*; n, *NcoI*; p, *PstI*; r, *RsaI*; e, *EcoRI*; s, *SalI*; sp, *SphI*.

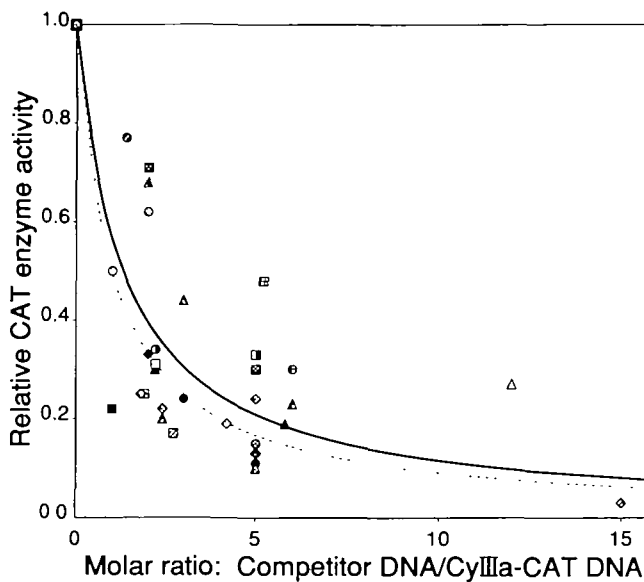


Fig. 2. Competition *in vivo* between the CyIIIa 5'-regulatory domain and coinjected CyIIIa · CAT reporter genes. At least 1200 molecules of a CyIIIa · CAT gene fusion were injected at each point, together with varying numbers of either the 9.5 kb *SaII* DNA fragment or the 4.3 kb *NcoI-EcoRI* DNA fragment, containing all known sites of *in vitro* interaction between the CyIIIa 5'-regulatory region and nuclear factors (see Fig. 1B) as competitor. The molar ratio of competitor sequence to CyIIIa · CAT sequence incorporated at each point was measured by slot-blot hybridization of DNA extracted from samples of pooled transgenic embryos, using ^{32}P -labeled probes specific for each sequence, as described in Materials and methods. A second aliquot of embryos was pooled and assayed for CAT activity at each point. The relationship between CAT activity per embryo relative to the CAT activity per embryo (normalized to 1.0) in the control in which no competitor DNA was included (y), and molar ratio, (x), of competitor DNA molecules to CyIIIa · CAT genes, can be approximated as $y = (1 + \alpha x)^{-1}$, where α is the competition efficiency coefficient, and provides an estimate of the fraction of competitor fragments actively functioning as competitor (Livant *et al.* 1988; see legend to Table 1). The dashed curve indicates the ideal form, i.e. where $\alpha = 1.0$. The solid curve is the least squares fit to the experimental data, and yields a value for α of 0.76 [Livant *et al.* (1988) obtained $\alpha = 0.44$ in similar experiments; considering the scatter there may be no real difference between these results, nor any real discrepancy from an α value of unity]. Twenty-six experiments are shown. Each unique symbol represents an experiment carried out with a single batch of eggs. CyIIIa · CAT gene fusion and competitor DNA fragment used are: solid symbols (black or white), 6.3 kb *SphI-KpnI* pCyIIIa · CAT · 1 fragment plus 9.5 kb *SaII* competitor DNA fragment; cross-hatched symbols, *SphI*-linear CyIIIa · CAT (Flytzanis *et al.* 1987) plus 9.5 kb *SaII* competitor DNA fragment; right-shaded symbols, *SphI*-linear CyIIIa · CAT plus 4.3 kb *NcoI-EcoRI* competitor DNA fragment.

mass of competitor DNA added to obtain the nominal competitor/fusion gene molar ratio desired. The total DNA mass injected was about 0.2 pg/egg.

As in our previous studies (*op cit*) average CAT enzyme content per embryo in the reference samples that contained no competitor was generally $1.0\text{--}3.0 \times 10^7$ enzyme molecules per embryo. To pool the data shown in Fig. 2 these values have been used for normalization of the CAT enzyme content in the embryos within each experimental series. The CAT enzyme was measured in samples of 24 h embryos, and in Fig. 2 is expressed as a function of the molar ratio of competitor to CyIIIa · CAT genes actually incorporated per average embryo. These ratios were directly measured in aliquots of the same embryo samples by slot-blot hybridization, using competitor DNA and CAT DNA sequence probes. The experimental data fit by the solid line in Fig. 2 indicate that CAT activity falls off at a rate that is almost stoichiometric, with respect to the number of competing 5' regulatory sequences present. The best fit value from the pooled data of Fig. 2 yields $\alpha = 0.76$ (see legend of Fig. 2). Thus we have used $\alpha = 0.76$ as a normalization factor in most of the following experiments. These data clearly confirm that endogenous factors required for the expression of the CyIIIa · CAT fusion gene can be titrated away from the fusion gene by introduction of excess DNA binding sites.

Competitive titration in vivo by subelements of the CyIIIa regulatory domain

To determine the effects on CyIIIa · CAT gene activity of titrating away those factors binding to individual subregions of the large regulatory domain, we coinjected various molar excesses of the subfragments identified in Fig. 1B. Many of these subfragments were also utilized for the gel-shift experiments of Calzone *et al.* (1988) by which the sites of interaction were initially located, and we have adopted the same subfragment nomenclature in the present account. Several include only a single site of DNA-protein interaction. However, the P1, P2 and P6 subfragments contain two sites of interaction, and the P8 subfragment, which is particularly complex, includes at least 8 protein-binding elements of three different classes (Thézé *et al.* 1990). Two subfragments that do not contain sequences that form DNA-protein complexes *in vitro* on reaction with embryo nuclear extracts are also shown on the map (X in Fig. 1B). Neither of these subfragments displayed any competitive function in our hands (results not shown), nor do subfragments extending 5.3 kb upstream of the *SphI* site (see Fig. 1A). In initial studies we found that microinjection of small DNA fragments (<1 kb) does not reliably result in measurable replication during embryogenesis, probably due to failure to form concatamers that are sufficiently large to promote stable incorporation. To circumvent this problem each of the competitor DNA subfragments shown in Fig. 1B, with the exception of the large subfragment containing the whole P8 binding region, was first ligated *in vitro* to carrier genomic DNA. The actual concentration of the specific competitor sequence in each ligated DNA

preparation was measured by slot-blot hybridization, using a ^{32}P -labeled RNA probe.

In each injection series, eggs obtained from a single female were coinjected with at least 1200 molecules of a CyIIIa·CAT gene, together with increasing quantities of competing sequence that were always in molar excess with respect to the CyIIIa·CAT DNA. Competition curves obtained *in vivo* for each of nine nonoverlapping subfragments that were derived from the CyIIIa regulatory domain are shown in Fig. 3. The average CAT enzyme content and the average competitor DNA: CyIIIa·CAT DNA ratio were measured in pooled embryo samples at 24 h. The average exogenous DNA amplification in the various injection samples *within* a given experiment was similar, and in different batches the values measured in the 24 h embryos ranged from about a 10-fold to an 80-fold increase over the amount of DNA injected per egg. Normalized experimental data were fitted to the same form describing the competition stoichiometry as above, and for each experiment a value for the competition efficiency coefficient α was derived by nonlinear least squares. In Fig. 3, competition functions are shown for each of 10 subfragments that contain known sites of DNA-protein interaction identified *in vitro*. These are presented in order of descending α values; i.e. the best competitors first, and the least effective last. The curve representing perfect competition stoichiometry ($\alpha=1$) is shown as a dotted line in each panel for comparison. Fig. 3A–F demonstrates immediately that a majority of the non-overlapping subfragments tested function individually as competitors against the whole CyIIIa regulatory domain, and the P4, P5 and P8 subfragments *each* appears to compete as well as does the whole regulatory domain. Each of these regions apparently contains an essential positive regulatory site(s) bound by a factor or factors of limited availability relative to the number of competitor fragments incorporated. Fig. 3G–J shows that subfragments containing the binding regions for P6, P3, P3A and P7II, respectively, displayed relatively insignificant competitive activity.

Quantitative data for these and some additional experiments are listed in Table 1. The values given by the parameter α' in the second column of Table 1 estimate the competitive effect of the indicated subfragment in each experiment, relative to the competition obtained with the *complete* regulatory region. Pooled data for multiple experiments with given subfragments were used to assess the best overall value of the competition coefficient, given as α'' in the third column of Table 1. Each of five different regions, i.e. those containing binding sites for P2, P4, P5, P7I and P8, compete between 10% and 100% (or better) than does the whole regulatory domain (i.e. $\alpha' \geq 0.1$). An α' value of 1.0 would indicate that the competitive effect of the subfragment is equal to that of the fragment containing the entire regulatory domain. The α' values for DNA subfragments containing the binding sites for P3, P3A, P6 or P7II are below 0.1, and that for P1 is just above 0.1. As discussed below, this result is unlikely to be due simply to high prevalence of the respective factors.

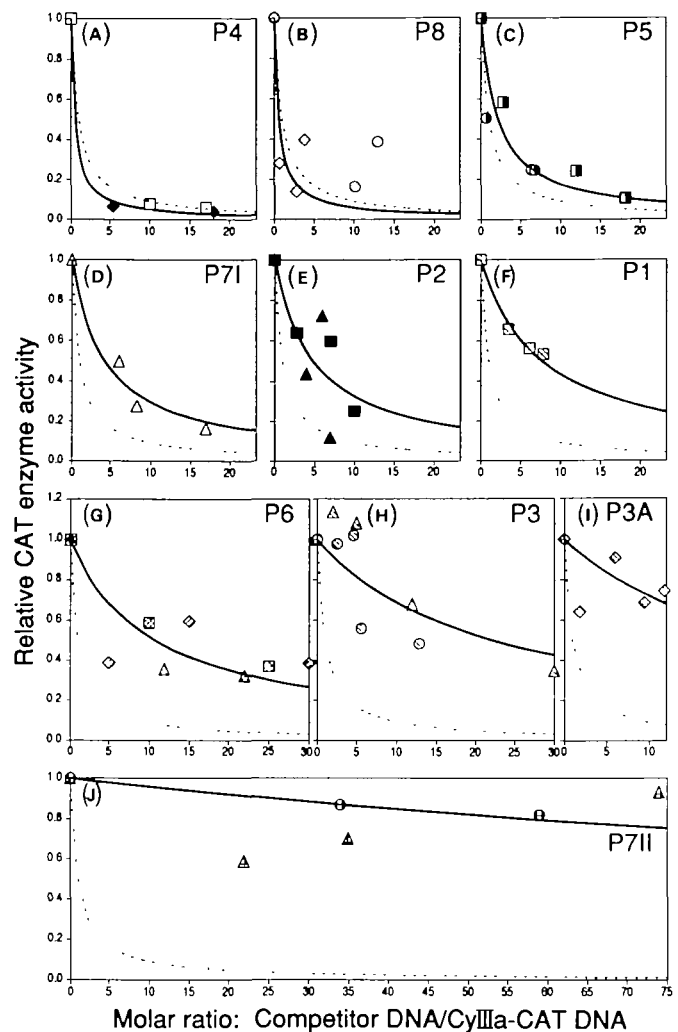


Fig. 3. *In vivo* competition by subelements of the CyIIIa 5'-regulatory region in transgenic embryos. At least 1200 molecules of a CyIIIa·CAT gene fusion were injected at each point, together with varying numbers of competitor fragments that contain the binding regions for the indicated set of DNA-binding factors (Thézé *et al.* 1990; see Fig. 1B). The relationship between average CAT activity per embryo and the average molar ratio of competitor DNA molecules to CyIIIa·CAT molecules incorporated is expressed as in Fig. 2. The competition coefficient α (i.e. where ideal stoichiometric competition would generate a value for α of 1.0) calculated for the whole regulatory domain in Fig. 2 was used as a normalization factor to correct the individual data points in these experiments (see legend to Table 1), so that the competitive effect of the individual fragments would be compared directly to the effect of the whole domain. Thus, the dashed curves in A–J indicate the competitive behavior of the entire regulatory region. The solid curves, fit by least squares, indicate the behavior of the individual subfragments. Competition is near stoichiometric in A–C. There is little, if any, competition in F–J. Panels A–C, E, G, H, J show the least squares fit to the pooled experimental data obtained from more than one experiment. The data for the eighteen experiments shown here were derived from a subset of those experiments used to generate the value of α for the whole regulatory domain shown in Fig. 2 (data points from a single experiment are described by matching symbols in these figures).

Table 1. Competitive effect in vivo of subfragments of the *CyIIIa* 5'-regulatory domain

Competitor CyIIIa subfragment*	Experiment	α' †	α'' ‡
P2	1	0.17 (0.11)	0.21 (0.19)
	2	0.25‡ (0.24)	
P4	1	2.5 (0.01)	1.8 (0.02)
	2	1.1 (0.1)	
P5	1	0.29 (0.04)	0.47 (0.12)
	2	0.92 (0.10)	
P7I	1	0.24 (0.7)	
P8	1	0.24 (0.14)	1.7 (0.22)
	2	2.2 (0.18)	
P1	1	0.13 (0.03)	
P3	1	0.02§ (0.04)	0.04 (0.18)
	2	0.14§ (0.09)	
	3	0.04 (0.17)	
	4	0.06 (0.17)	
P3A	1	0.04 (0.16)	
P6	1	0.09‡ (0.21)	0.09 (0.15)
	2	0.07‡ (0.00)	
	3	0.12 (0.05)	
P7II	1	0.01 (0.23)	0.04 (0.17)
	2	0.003 (0.06)	

* Each competitor subfragment is derived from the entire *CyIIIa* 5'-regulatory domain, and contains the binding regions for the sets of DNA-binding factors indicated in Fig. 1B (Thézé *et al.* 1990; Calzone *et al.* 1988).

† The relative activities of the *CyIIIa* · CAT reporter gene in the presence of molar excesses of the indicated competitor DNA subfragment in each experiment were normalized, usually by the value for the competition efficiency coefficient estimated from the pooled control data shown in Fig. 2. This value was $\alpha=0.76$. To carry out the normalizations the following procedure was used. y_i is the CAT activity measurement (average molecules CAT enzyme/embryo in the competition sample, relative to the average molecules CAT enzyme/embryo in the control sample in which no competitor DNA was included; see Materials and methods), x_i is the molar ratio [subfragment:*CyIIIa* · CAT] actually incorporated, by direct measurement (see Materials and methods); N is the competition efficiency coefficient indicating the fraction of competitor functioning in a manner quantitatively equivalent to the respective site(s) on the incorporated *CyIIIa* · CAT molecules. In general for this competition, $y=(1+\alpha x)^{-1}$ (Livant *et al.* 1988). The normalized values denoted $y(c;x_i)$ are calculated as follows:

$$y(c;x_i) = y_i \cdot \frac{y(1;x_i)}{y(N;x_i)}$$

where N is the value of α used for the normalization, and $y(1;x_i)=(1+x_i)^{-1}$; $y(N;x_i)=(1+Nx_i)^{-1}$. As noted above N was usually 0.76, except for a few cases, indicated (see below) in which there were sufficient internal control data, so that N was derived from that experiment rather than from the pooled data. The resulting data reduction in these cases was in fact never significantly dependent on whether the internal control or the pooled value 0.76 were used for the normalization. Internal controls were routinely used as a qualitative screen for occasional batches of eggs in which for some reason even the control competition functioned not at all or very poorly, probably because these eggs did not adequately support exogenous DNA amplification. The normalized $y_c(x_i)$, x_i values were fit to $y_c(x_i)=(1-\alpha'x_i)^{-1}$ by least squares, and the values of α' are those reported in the Table for the individual subfragment experiments. For the last column of Table 1 all of the $y_c(x_i)$ values were pooled, the data were refit, and an α'' value established by least squares. Root mean square errors are reported in parentheses.

‡ For these experiments, we used individually determined efficiency coefficients to obtain α' , rather than 0.76 (i.e., individually determined values of N , as in note †; here the value for α for the *CyIIIa* · CAT control with the whole regulatory domain competitor in each of these particular experiments). For P2, experiment 2, $N=0.96$; for P6, experiment 1, $N=0.73$; P6, experiment 2, $N=0.31$.

§ Measurements of CAT DNA and competitor DNA content per embryo were not made in these experiments. The values calculated for α' were estimated based on the subfragment/CAT DNA molar ratios introduced into each egg. These experiments were not utilized in calculating α'' .

Lethal effects of competitive titration of the P3B binding site

The site labeled P3B in Fig. 1 includes a canonical octamer binding site (Thézé *et al.* 1990). Uniquely among all the subfragments tested, competition with a subfragment containing only this site results in the death of 60 % to over 95 % of the recipient embryos, by the pluteus stage, in different experiments. In controls for these particular experiments, 95–100 % of eggs injected only with a saturating number of *CyIIIa* · CAT molecules plus carrier DNA developed through hatching blastula stage, and 70–80 % completed embryogenesis normally. The same P3B injection solution that is lethal to recipient embryos is rendered harmless if treated with DNase prior to injection.

The unusual lethality of P3B competition is particularly interesting because as shown earlier the adjacent P3A site, when injected on a fragment by itself, does not display competitive activity (Fig. 3I; Table 1), and when the *HindIII*–*DdeI* 'P3' subfragment, which includes both the P3A and P3B sites (see Fig. 1A) is injected, the results are the same as when the P3A subfragment alone is injected (see Table 1). That is, there is no significant competitive activity (α' is about 0.04 for both subfragments), and no lethality is observed. The P3B factor is evidently required for the expression of genes that are necessary for viability, a not unlikely possibility since octamer binding sites have been reported in a great many different genes in mammals, and in several sea urchin histone genes as well (Barberis *et al.* 1987). That the presence of the P3A site on the same competitor fragment with the P3B site 'rescues' the embryos from the lethal effects of P3B competition suggests that, when P3A is bound at the adjacent sites, the essential P3B octamer factor is precluded from binding, and thus is not sequestered away on the competitor fragments.

Discussion

Competitive titration of positively acting factors within the embryo nuclei

Livant *et al.* (1988) showed that as the number of *CyIIIa* · CAT genes per embryo nucleus is increased, over a range from a few to several thousand, the amount of CAT enzyme expressed attains a plateau, after an initial phase in which CAT activity rises proportionally with incorporated gene number. The kinetics of the response can be fitted to the expected form for a saturation phenomenon. This is an interesting result which it is necessary to consider briefly before taking up the nature of the competitions that we present in this paper. About 90 % saturation of *CyIIIa* · CAT expression occurs at 1050 specific sites per nucleus, implying that the limiting factors required for *CyIIIa* · CAT expression are 90 % bound to specific sites at this level (D. Livant and E. Davidson, unpublished). In the present experiments, we tried to approach saturation conditions by injecting ≥ 1100 molecules of *CyIIIa* · CAT per egg, plus in the competition samples, molar

excess of the individual sites being tested. On the saturation plateau, the excess sites compete with one another for factor molecules. As the site concentration increases in the nuclei, these factors progressively repartition from the non-specific sites with which they are reversibly associated to the added specific sites, according to the particular values of the partition function K_r that obtain for each factor *in vivo* (K_r is the ratio of equilibrium constants for reaction of a given factor with its specific sites to that for reaction with non-specific DNA sites). Cooperative binding amongst the many different factors required for productive expression of CyIIIa·CAT, or additional stabilization of active transcription complexes, is also implied. Otherwise, as excess copies of the regulatory domain are introduced, the factors would distribute independently amongst them. The number of completely loaded regulatory domains would thus decrease, and a decline in activity rather than the saturation plateau observed convincingly by Livant *et al.* (1988) would result.

The experiments shown in Fig. 2 confirm the evidence of Livant *et al.* (1988) that when the whole regulatory domain is introduced in molar excess with respect to saturating levels of CyIIIa·CAT, competition for necessary factors occurs *in vivo*. This is reflected by an almost stoichiometric decrease in CAT expression, as a function of competitor: CyIIIa·CAT molar ratio. When similar competition is observed following introduction of molar excesses of a given site, as in the experiments of Fig. 3, these competitor sites therefore must effectively titrate away the factor species that recognizes them specifically. However, we have not shown explicitly that the competitive effects are direct. For example the striking P5 competition shown in Fig. 3C could be an indirect result, were the sequestration of the P5 factor actually to affect some regulatory gene, that in turn produces some other factor that affects CyIIIa·CAT expression. The improbability of such an interpretation depends on the frequency with which factors required for regulation of the CyIIIa gene are also required for regulation of genes whose products are necessary for CyIIIa expression, e.g. these same factors, or polymerase components, aspects of processing mechanism, etc. Needless to say, that frequency cannot yet be assessed. But, it is worth noting that in two cases where there is relevant deletion evidence, the same result is obtained by both deletion and competition methods. Flytzanis *et al.* (1987) showed that severe depression of CyIIIa·CAT expression results from excision of the P8 sites (their ΔSP , ΔSPP deletions), or of the P5 site (their ΔH deletion, in which exactly the same fragment was removed from CyIIIa·CAT as was used for the experiments of Fig. 3C). It follows that the strong competitions shown for the P5 and P8 sites in Fig. 3B and 3C are indeed direct rather than indirect effects. More generally, however, it is useful to recognize that excess site competition, and site deletion followed by gene transfer, are complementary rather than redundant ways of probing the significance of regulatory interactions *in vivo*. Deletion does indeed reveal site function directly,

but in the context of more or less disturbed *cis*-regulatory DNA structure. While this may usually be of secondary or no importance, interaction between bound factors could depend in given cases on regulatory domain structure. In one example, a cardiac myosin light chain gene in which the relevant sites are closely apposed, intranuclear competitive titration yielded consistent results where deletion did not (Braun *et al.* 1989). Recently competitive titration has been used in studies of the regulatory molecular biology of sea urchin embryo histone genes (Lai *et al.* 1989), human actin genes (Muscat *et al.* 1988) and a gut-specific esterase gene of *C. elegans* (McGhee, personal communication).

Identification of positively acting CyIIIa regulatory sites

The surprising result from the experiments shown in Fig. 3 and Table 1 is that out of ten subfragments tested five include sites that compete effectively at low molar excess with respect to CyIIIa·CAT expression. These are, in order of their competitive potency, the subfragments containing the P4, P8(I+II+III), P5, P7I and P2(I+II) sites. In addition, the P1 factor may compete weakly and P3B octamer factor is clearly necessary, though its role in CyIIIa gene regulation remains unspecified, since its sequestration in a minor fraction of cells is lethal to the embryos. The positively acting factors listed here are probably entirely different and independent of one another, since the sequences to which they bind are wholly distinct (Thézé *et al.* 1990), and *in vitro* measurements indicate that these factors are of different prevalences (Calzone *et al.* 1988). Furthermore, these particular factors accumulate in the embryo with different kinetics (Calzone *et al.* 1988), and interact with different unique sets of genes (Thiebaud *et al.* 1990). The three most potent competitors are those subfragments bearing sites for factors P4, P8(I+II+III), and P5. In all of these cases, the individual subfragment competes within a factor of two as efficiently as does the whole regulatory domain. Considering the statistical nature of these observations, and the failure of the whole domain to compete ideally ($\alpha=0.76$; Fig. 2), it seems reasonable to conclude that the competitive efficiencies of these subfragments cannot be distinguished from that of the whole regulatory domain. Thus, each of these subfragments must be the site of absolutely required, positive interactions, without which CyIIIa·CAT activity is minimal. The prevalence of the P4, P8 and P5 factors in the embryo nuclei is close to that of the CyIIIa·CAT gene introduced in our competition experiments (i.e. ~500 molecules/nucleus of P4 and P5, and about 1500 of the P8 factors). This prevalence will be rapidly exceeded as molar excesses of competitor sites are introduced. P4 is a CCAAT factor and the essentiality of this interaction is thus not surprising, though it may be noted that there are probably several different CCAAT binding factors in sea urchin embryos (Barberis *et al.* 1987). Although molar excesses of sites should also have been attained *in vivo* for the relatively rare P7I factor (~200 copies/

nucleus) and for P1 (~1100 copies/nucleus), competition in these cases is only around one-fourth to one-sixth as efficient as with the whole domain. Unless the P1 and P7I factors are distributed to only a small subset of cells, in which their concentrations per nucleus would be 5–10× higher, it can be concluded that these interactions are not individually absolutely required for CyIIIa·CAT function, as are those mediated by the sites on the P4, P8 and P5 subfragments. Yet they are necessary for maximum activity.

There can be two reasons for failure of competition when competition sites are introduced: factors with relatively low K_r will not partition to the added sites efficiently; and for factors that are very prevalent it is more difficult to introduce a true excess of specific sites. High endogenous factor prevalence (~6000 copies/nucleus) could easily explain the relatively weak competition displayed by the P2 subfragment. The number of CyIIIa·CAT genes plus competitor would only begin to exceed the number of P2I and P2II factor molecules at the highest molar excesses utilized (cf. Fig. 3E). Thus it cannot be excluded that one or both of the P2 factors is not as essential as at least one of the P8 and the P4 and P5 factors; that is, that the efficiency of P2 subfragment competition is depressed to about one-fifth of the competition efficiency of the whole domain only because of failure to achieve site/factor ratios equivalent to those in the other experiments shown. It is not feasible to introduce greater masses of DNA without incurring risk of embryo lethality irrespective of the sequence injected (McMahon *et al.* 1985). On the other hand, the almost complete failure of the P3A, P6 and P7II subfragments to compete effectively cannot be explained simply in the same way. The average numbers of molecules of these factors per nucleus are not very different than those for which clear competition was obtained (P3A, ~720 molecules/nucleus; P6, ~2800 molecules/nucleus; P7II, ~1480 molecules/nucleus; Calzone *et al.* 1988). Though the K_r values of these factors differ (Calzone *et al.* 1988), they are all sufficiently high so that competition should have been observed at the molar ratios actually achieved, were these positively acting factors required for embryonic CyIIIa·CAT expression.

The CyIIIa regulatory domain is a logic switch

Interference with the interactions so far discussed depresses CyIIIa·CAT function *in vivo*, but in all cases the residual expression is spatially correct. As shown in the accompanying paper (Hough-Evans *et al.* 1990), in embryos with reduced CyIIIa·CAT function due to *in vivo* titration, CAT mRNA is still confined to aboral ectoderm cells. While these interactions are all positive in sense, the P3A and P7II subfragments, which do not competitively titrate down CyIIIa·CAT activity (Fig. 3 and Table 1), function *in vivo* as negative regulators of spatial expression. Viewed as a functional unit, the CyIIIa regulatory domain can be seen to require that a number of distinct factor-site interactions take place in order for the gene to function properly in time, in space (i.e. cell lineage) and in amplitude. The inference from

what we are learning about this developmental gene regulatory system is that the multiple interactions it requires are not, as may appear superficially, redundant in a functional sense. They exist to carry out *diverse biological functions*. Perhaps each bears a specific functional relation to the biological status of the cell, that is, to developmental stage, state of specification or lineage, etc. In the case of the P8 and P5 factors, for example, which seem at first sight to function redundantly, i.e. as essentially positive regulators (this paper), we have good reason to suspect quite different biological roles. The P8 factors are present in the embryo before the CyIIIa or CyIIIa·CAT genes are activated, and their concentration remains unchanged as development proceeds (Calzone *et al.* 1988). Thus they are unlikely to be responsible for temporal activation, but to be needed as amplitude controllers. On the other hand, the same study shows that the P5 factor rises dramatically over the same period in which this and other aboral ectoderm genes are turned on (Davidson, 1989). P5 could control this important temporal developmental function, since it too is essential for expression, and this factor is probably utilized in the regulatory domains of several other genes that are activated at the same stage of development (Thiebaud *et al.* 1990). Thus we think of the CyIIIa regulatory system as a developmental logic switch, in the sense that it converts the multielement pattern of individual, functionally diverse interactions within it, into a single scalar function, *viz.* increase (or decrease) in the rate of transcriptional expression.

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